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Clarification of the Taxonomy of *Bacillus mycoides*

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Because of the very similar physiological properties and base sequences of the 16S rRNAs of *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis*, some taxonomists question the validity of separating these organisms into distinct species. DNA relatedness studies based on spectrophotometrically measured renaturation rates were carried out to determine the taxonomic relationships of the three species. A study of 58 strains revealed that the levels of relatedness between *B. cereus* and *B. mycoides* and between *B. cereus* and *B. thuringiensis* ranged from 22 to 44% and from 59 to 69%, respectively. On the basis of the moderately high levels of DNA relatedness which we determined, *B. cereus* and *B. thuringiensis* appeared to be genetically related but taxonomically distinct entities. The *B. mycoides* group was genetically distantly related to the *B. cereus* group and represented a separate taxon. Furthermore, our data indicated that the *B. mycoides* group consists of two genetically distinct groups, each of which represents a distinct species. In addition to rhizoidal colonial morphology and lack of motility, the *B. mycoides* group could be distinguished from *B. cereus* by differences in fatty acid profiles and acetanilide-producing activities.

The species *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis* were included on the 1980 Approved Lists of Bacterial Names (13). The characteristics used to differentiate these taxa are pathogenicity and gross morphological characteristics; *B. anthracis* and *B. thuringiensis* are mammalian and insect pathogens, respectively, while the saprophytic organism *B. mycoides*, in contrast to *B. cereus*, produces rhizoidal colonies. Because these four species share many phenotypic characteristics, some taxonomists have questioned their status as separate species (4, 14). The results of DNA relatedness studies appear to confirm the close taxonomic relationship of *B. anthracis*, *B. cereus*, and *B. thuringiensis* (6, 9, 12). Limited data have suggested that *B. mycoides* may also be genetically closely related to *B. cereus* (12, 16).

Recently, Ash et al. (1) have shown that these four species exhibit >99% similarity in their 16S rRNA base sequences. Although the sequence data suggest that the four taxa are closely related genetically, they do not necessarily refute the theory that four separate species exist. Several genetically distinct species (2, 17) that exhibit sequence similarity values comparable to those found by Ash et al. (1) are known. In order to resolve the taxonomic relationships of these organisms, we evaluated the DNA relatedness of *B. cereus*, *B. mycoides*, and *B. thuringiensis*.

MATERIALS AND METHODS

Organisms. Table 1 lists the *Bacillus* strains used in this study. These strains are maintained in the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research. Each Northern Regional Research Laboratory (NRRL) designation includes the prefix B-, which designates strains acquired directly from individuals or strains isolated at the National Center for Agricultural Utilization Research, the prefix NRS-, which identifies strains obtained from the *Bacillus* collection of N. R. Smith deposited in the Agricultural Research Service Culture Collection by R. E. Gordon, the prefix HD-, which indicates strains coming from H. Dulmage's collection of *B. thuringiensis*, or the prefix BD-, which labels strains obtained from B. Delaporte. Working stock cultures were grown at 28°C on nutrient agar amended with 5 mg of $MnSO_4 \cdot H_2O$ per liter until sporulation occurred and were stored at 4°C.

DNA isolation and reassociation. For DNA isolation, the organisms were grown in TGY broth (5) with agitation at 28°C and were harvested by centrifugation at 5°C in the mid- to late-logarithmic growth phase when microscopic examination revealed an absence of sporulation.

DNA was extracted and purified by a modification of method of Marmur (8). The modification involved using CsCl ultracentrifugation (7) to produce highly purified DNA samples. The purity and quality of each DNA preparation were such that the A_{260}/A_{280} and A_{260}/A_{230} ratios were consistently 1.8 to 1.9 and 2.0 to 2.3, respectively. The quality of the DNA preparations was confirmed from melting curves that showed hyperchromicities ranging from 38 to 40% (8).

The procedure used to estimate the extent of reassociation spectrophotometrically has been described previously (10). The extent of reassociation was calculated by the equation of De Ley et al. (3).

Fatty acid analysis. The whole-cell fatty acid contents of *Bacillus* strains were determined by using the MIDI system of Sasser (11). Because the tenacious adherence of *B. mycoides* strains to agar surfaces interfered with collection of cells, the cultures used for fatty acid analysis were grown in Trypticase soy broth for 16 h at 28°C with agitation at 200 rpm. To ensure nonbiased comparisons, strains of *B. cereus* and *B. thuringiensis* were grown under identical conditions.

Numerical analyses. Clustering of DNA relatedness values based on the unweighted pair group arithmetic average algorithm (15) was carried out by using the PC-SAS version 6.04 (SAS Institute, Inc., Cary, N. C.) SAS/STAT cluster procedure. A dendrogram was generated with SAS/GRAPH, using the SAS macro GRAFTREE written and kindly provided by Dan Jacobs, University of Maryland. The SAS analyses were performed with a DTK-486 computer.

Acetanilide production. All of the strains were grown for 18 to 24 h in a medium containing (per liter of deionized water) 20 g of glucose, 5 g of yeast extract, 5 g of tryptone, and 1 g of K_2HPO_4. For the assays, 5-ml cell suspensions were treated with 25 μl of aniline and shaken at 250 rpm and 28°C for 24 h. Thin-layer chromatography was performed on Silica Gel 60 F-254 by using a mobile phase consisting of benzene, ethanol, and acetic acid (80:20:0.4). Acetanilide and aniline appeared as fluorescent spots when the gels were observed under UV light. High-pressure liquid chromatography analyses were performed by using a Spectra-Physics model SP8700 pump and a model SP8440 UV-visible light detector operating at 245 nm. Components obtained from the assays were separated on a Waters μBondapak C18 column by using a 50% aqueous methanol mobile phase at a flow rate of 1 ml/min.

Acetanilide was isolated from the reaction mixtures by extraction into ethyl acetate. After decolorization with activated carbon and drying with sodium sulfate, the ethyl acetate was removed under reduced pressure; the final product was a dull yellow oil.

RESULTS

Tables 2 through 4 show the levels of DNA relatedness of *B. cereus*, *B. mycoides*, and *B. thuringiensis* strains. According to our data, the levels of DNA relatedness between NRRL B-3711, the type strain of *B. cereus*, and several *B. cereus*, *B. thuringiensis*, and *B. mycoides* strains ranged from 74 to 100%, from 59 to 69%, and from 25 to 44%, respectively; the levels of DNA relatedness between *B. thuringiensis* reference strain NRRL HD-4 and several *B. cereus*, *B. thuringiensis*, and *B. mycoides* strains ranged from 59 to 67%, from 60 to 69%, and

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TABLE 1. Strains used in this study

Strain	Received as:	Source[a]	History
B. cereus strains			
NRRL B-569	8b	1	K. B. Raper
NRRL B-1530		2	N. Bohonos ← S. A. Waksman strain O
NRRL B-1868	M8	3	J. R. Norris (M.17)(8)2ATY
NRRL B-1877	NRS-768	4	G. Knaysi
NRRL B-2915	NRS-1122	4	ATCC 9620
NRRL B-3439	7	5	*Bacillus popilliae*
NRRL B-3711[T][b]	ATCC 14579[T]	6	R. E. Gordon [= DSM 31[T]][c]
NRRL B-4288	2173	7	
NRRL B-4552		1	G. St. Julian
NRRL B-4556	ATCC 21281	6	Seibu Chemical Co., Ltd.
NRRL B-14145	H3548	8	Tapai-ubi isolate
NRRL B-14721	ATCC 33019	6	Torres-Anjel ← H. E. Giralds ← G. H. Gonzales; enterotoxigenic
NRRL B-14725	DSM 2302	9	R. Holbrook ← R. T. Gilbert; enterotoxigenic [= CBCC 2823]
NRRL B-14727	DSM 4384	9	E. A. Johnson B4ac; enterotoxigenic
NRRL NRS-204	NRS-204	10	N. R. Smith; soil isolate
NRRL NRS-205	NRS-205	10	N. R. Smith; soil isolate
B. thuringiensis strains			
NRRL HD-2	HD-2	11	H. de Barjac; serovar thuringiensis
NRRL HD-3	HD-3	11	H. de Barjac; serovar finitimus
NRRL HD-4	HD-4	11	H. de Barjac; serovar alesti
NRRL HD-5	HD-5	11	H. de Barjac; serovar kenyae
NRRL HD-7	HD-7	11	H. de Barjac; serovar dendrolimus
NRRL HD-9	HD-9	11	H. de Barjac; serovar entomocidus
NRRL HD-11	HD-11	11	H. de Barjac; serovar aizawai
NRRL HD-12	HD-12	11	H. de Barjac; serovar morrisoni
NRRL HD-146	HD-146	11	H. de Barjac; serovar darmstadiensis
NRRL HD-224	HD-224	11	H. de Barjac; serovar canadensis
NRRL HD-567	HD-567	11	H. de Barjac; serovar israelensis
B. mycoides group 1 strains			
NRRL B-347	NRS-911	4	J. R. Porter ← O. F. Edwards ← H. J. Conn
NRRL B-615	NCH5	1	L. B. Wickerham; isolated from soil
NRRL B-3436	447D	1	E. Afrikan
NRRL B-14811[T]	DSM 2048[T]	9	ATCC 6462[T] ← N. R. Smith 273[T]
NRRL BD-2		12	M. Guilliermond
NRRL BD-3		12	M. R. Legroux
NRRL BD-4	X.42	12	Soil isolate
NRRL BD-7	6.A.4	12	Soil isolate
NRRL BD-9	9.A.1	12	Soil isolate
NRRL BD-12	9.A.9	12	Soil isolate
NRRL BD-15	10.G.1	12	
NRRL BD-18	18.B.8	12	Soil isolate
NRRL BD-23	58.C.2	12	Soil isolate
NRRL NRS-273[T]	NRS-273[T]	10	N. R. Smith 155; soil isolate [= ATCC 6462[T]]
NRRL NRS-306	NRS-306	10	M. H. Soule [= ATCC 6463]
NRRL NRS-319	NRS-319	10	N. R. Smith L2B3; soil isolate
NRRL NRS-325	NRS-325	10	N. R. Smith C2B4; soil isolate
NRRL NRS-1316	NRS-1316	10	B. Delaporte 6.A.4
B. mycoides group 2 strains			
NRRL B-346	NRS-233	4	AMNH ← W. W. Ford and J. S. Lawrence
NRRL B-617		1	L. B. Wickerham; soil isolate
NRRL B-618		1	L. B. Wickerham; soil isolate
NRRL BD-5	6.A.1	12	Soil isolate
NRRL BD-6	6.A.3	12	Soil isolate
NRRL BD-10	9.A.3	12	Soil isolate
NRRL BD-14	9.S.2	12	Soil isolate
NRRL NRS-318	NRS-318	10	N. R. Smith L2B2; soil isolate
NRRL NRS-321	NRS-321	10	N. R. Smith C2B6; soil isolate
NRRL NRS-322	NRS-322	10	N. R. Smith C2B1; soil isolate
NRRL NRS-323	NRS-323	10	N. R. Smith C2B2; soil isolate
NRRL NRS-324	NRS-324	10	N. R. Smith C2B3; soil isolate
NRRL NRS-327	NRS-327	10	N. R. Smith Iowa 1; soil isolate
NRRL NRS-371	NRS-371	10	N. R. Smith; soil isolate
B. mycoides NRRL NRS-1216 (no group)	NRS-1216	10	H. W. Reuszer Army 929

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[b] T = type strain.

[c] Designations in brackets are equivalent designations. DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; CBCC, Colworth Bacterial Culture Collection; ATCC, American Type Culture Collection.

TABLE 2. Levels of DNA relatedness between *B. cereus* strains and type or reference strains of *B. cereus*, *B. thuringiensis*, and *B. mycoides*

B. cereus strain	% Reassociation with DNA from[a]:		
	B. cereus NRRL B-3711[T]	*B. thuringiensis* NRRL HD-4[b]	*B. mycoides* NRRL NRS-273[T]
NRRL B-569	80	60	30
NRRL B-1530	100	66	32
NRRL B-1868	100	61	29
NRRL B-1877	100	59	25
NRRL B-2915	98	64	34
NRRL B-3439	88	69	27
NRRL B-4288	80	65	29
NRRL B-4552	81	66	24
NRRL B-4556	74	62	30
NRRL B-14145	89	65	33
NRRL B-14721	97	68	25
NRRL B-14725	85	67	31
NRRL B-14727	100	66	32
NRRL NRS-204	93	62	26
NRRL NRS-205	80	63	31

[a] The reassociation values are averages of the values from two determinations; the maximum difference in values between determinations was 6%.
[b] Reference strain.

22 to 36%, respectively; and the levels of DNA relatedness between *B. mycoides* type strain NRRL NRS-273 and selected strains of *B. cereus*, *B. thuringiensis*, and *B. mycoides* ranged from 24 to 34%, from 29 to 37%, and from 20 to 100%, respectively. The level of DNA relatedness between NRRL NRS-273 and NRRL B-14811, *B. mycoides* type strains obtained from two different sources, was 100%.

The dendrogram in Fig. 1 shows the levels of DNA relatedness among all 32 strains previously identified as *B. mycoides* and the *B. cereus* type strain (i.e., each strain was compared with all other strains). According to this dendrogram, the strains of *B. mycoides* which we examined segregated into two clusters that were related at a level of approximately 30%. Strain NRRL NRS-1216 was not closely related to *B. cereus* or *B. mycoides*; thus, it was improperly identified.

Table 5 shows that 12:0 iso, 12:0, 13:0 iso, 13:0 anteiso, 14:0 iso, 15:0 anteiso, 16:0 iso, 16:0, 17:0, and 17:0 anteiso fatty

TABLE 3. Levels of DNA relatedness between *B. thuringiensis* strains and type or reference strains of *B. cereus*, *B. thuringiensis*, and *B. mycoides*

B. thuringiensis strain	% Reassociation with DNA from[a]:		
	B. cereus NRRL B-3711[T]	*B. thuringiensis* NRRL HD-4[b]	*B. mycoides* NRRL NRS-273[T]
NRRL HD-2	65	66	30
NRRL HD-3	67	63	37
NRRL HD-5	60	64	29
NRRL HD-7	69	64	32
NRRL HD-9	59	69	34
NRRL HD-11	60	66	33
NRRL HD-12	65	62	29
NRRL HD-146	67	60	30
NRRL HD-224	66	60	31
NRRL HD-567	63	65	37

[a] The reassociation values are averages of the values from two determinations; the maximum difference in values between determinations was 6%.
[b] Reference strain.

TABLE 4. Levels of DNA relatedness between *B. mycoides* strains and type or reference strains of *B. cereus*, *B. thuringiensis*, and *B. mycoides*

B. mycoides strain	% Reassociation with DNA from[a]:		
	B. cereus NRRL B-3711[T]	*B. thuringiensis* NRRL HD-4[b]	*B. mycoides* NRRL NRS-273[T]
NRRL B-346	38	31	20
NRRL B-347	44	29	97
NRRL B-615	32	22	71
NRRL B-617	37	27	31
NRRL B-618	32	30	31
NRRL B-14811[T]	30	28	100
NRRL NRS-273	33	30	(100)[c]
NRRL NRS-318	30	36	39
NRRL NRS-319	28	30	96
NRRL NRS-321	25	30	32
NRRL NRS-1316	34	31	96

[a] The reassociation values are averages of the values from two determinations; the maximum difference in values between determinations was 6%.
[b] Reference strain.
[c] Parentheses indicate that by definition the reassociation value was 100%.

acids are the principal fatty acids found in the two *B. mycoides* groups and in *B. cereus*. Differences in the levels of 12:0 iso, 12:0, 13:0 anteiso, 15:0 iso, and 16:0 fatty acids differentiate *B. cereus* from the two *B. mycoides* groups, and differences in the levels of 12:0 and 13:0 anteiso fatty acids distinguish the two *B. mycoides* groups.

Interestingly, our studies revealed that 33 of 39 *B. cereus* strains and all 36 *B. thuringiensis* strains tested very efficiently acetylated aniline (i.e., transformed aniline to acetanilide

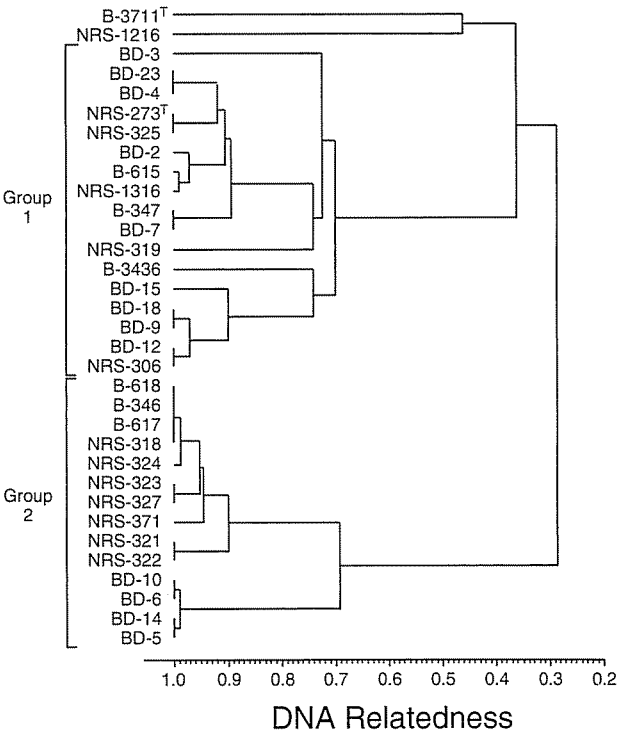

FIG. 1. Dendrogram showing levels of DNA relatedness among *B. mycoides* strains, based on unweighted average-linkage clustering. All strain designations are NRRL designations.

TABLE 5. Fatty acid profiles of *B. cereus* and *B. mycoides* groups

Taxon	No. of strains	Fatty acid composition (%)											
		12:0 iso	12:0	13:0 iso	13:0 anteiso	14:0 iso	14:0	15:0 iso	15:0 anteiso	16:0 iso	16:0	17:0 iso	17:0 anteiso
B. mycoides group 1	9	**2.9 ± 1.0**[a]	1.7 ± 0.7	23.1 ± 6.8	2.3 ± 0.5	5/3 ± 1.7	5.3 ± 2.3	22.9 ± 2.9	3.1 ± 0.8	4.7 ± 1.8	8.3 ± 2.1	7.1 ± 3.0	0.9 ± 0.4
B. mycoides group 2	7	**7.2 ± 1.8**	1.3 ± 0.4	17.0 ± 4.4	**6.1 ± 2.1**	3.5 ± 1.8	4.4 ± 0.8	19.3 ± 3.2	3.4 ± 0.8	4.6 ± 1.2	7.9 ± 1.7	8.8 ± 3.0	1.6 ± 0.5
B. cereus	8	**0.9 ± 0.3**	**0.2 ± 0.2**	11.5 ± 3.7	1.6 ± 0.4	5.2 ± 2.0	3.1 ± 0.7	**30.7 ± 2.8**	5.5 ± 1.6	5.3 ± 1.5	**3.9 ± 1.6**	7.2 ± 1.7	1.1 ± 0.3

[a] Mean ± standard deviation. Boldface type indicates significantly different values.

within 24 h) (data not shown). In contrast, strains of mesophilic spore formers, such as the two *B. mycoides* groups, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus laterosporus*, *Bacillus sphaericus*, *Bacillus megaterium*, and *Bacillus amyloliquefaciens*, did not acetylate aniline even after incubation for 96 h.

DISCUSSION

Ash et al. (1) reported that the levels of similarity of 16S rRNA sequences of *B. cereus*, *B. thuringiensis*, and *B. mycoides* were approximately 99.6%. These workers felt that such high levels of similarity alone were not necessarily indicators of conspecificity of the three taxa because of different rates of sequence divergence (1). There are other examples where distinct genospecies exhibit small numbers of nucleotide differences; these include members of the *Enterococcus avium* group (17), *Enterococcus casseliflavus*, and *Enterococcus gallinarum* (17), as well as *Aerococcus viridans* genospecies 1 and 2 (2). Ash et al. suggested that the specific relationships of *B. cereus*, *B. thuringiensis*, and *B. mycoides* could be resolved only by determining levels of DNA-DNA relatedness.

The DNA relatedness data obtained in this study supported previous suggestions (9) that *B. cereus* and *B. thuringiensis* are distinct but genetically moderately closely related species. Our data, however, clearly demonstrated that *B. cereus* and *B. thuringiensis* are not closely related genetically to *B. mycoides*. Hence, the present classification in which *B. mycoides* is recognized as an independent species is valid and should be retained. Furthermore, our data indicated that *B. mycoides* is a conglomerate of two genetically distinct groups; one of these groups is clustered around the type strain, and the status of the other needs to be determined. Our findings contradict the DNA reassociation data in studies of Kaneko et al. (6), Seki et al. (12), and Sommerville and Jones (16), which suggested that *B. mycoides* and *B. cereus* may be closely related genetically. However, the reliability of those studies is questionable because only a few poorly documented strains of *B. mycoides* were used.

In this study we also identified phenotypic characteristics other than gross morphology (rhizoidal colonial growth) and lack of motility (4) that differentiate *B. mycoides* from *B. cereus* and *B. thuringiensis*. The rhizoidal characteristic appears to be reasonably stable; mostly rhizoidal colonies appear when organisms are plated for observation of individual colonies. According to Gordon et al. (4), cultures maintained for up to 22 years in the laboratory have retained their unique character. However, nonrhizoidal colonial variants of *B. mycoides* have been observed (4). Frequent transfers with small inocula appear to result in selection of the nonrhizoidal variants and eventually produce nonrhizoidal cultures (4). *B. cereus* and the *B. mycoides* subgroups can be clearly distinguished by their distinctive fatty acid profiles. Finally, *B. cereus* and the closely related species *B. thuringiensis* appear to have the unique ability to acetylate aniline vigorously to form acetanilide. Acetanilide synthesis occurs rarely among *B. mycoides* strains. Preliminary data suggest that acetanilide production may be an additional characteristic that can be used to differentiate mesophilic aerobic spore formers.

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